INTERFERON PRODUCTION AS A MANIFESTATION OF DELAYED HYPERSENSITIVITY

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The most remarkable histologic feature of the inflammatory reaction produced in response to a viral infection is the localized accumulation of mononuclear cells, usually identified as lymphocytes and macrophages. This is true of the perivascular cuffing described for viral pneumonias or encephalitides as well as the more diffuse but still localized dermal reaction to vaccinia. Yet surprisingly little is known about what these cells are doing, especially when contrasted to the well defined functions of neutrophiles in the inflammatory response to bacterial infection. Virus-induced lesions are histologically similar to those of delayed hypersensitivity reactions; however, until recently, little had been added to this otherwise superficial relationship.

Recent developments in immunology, virology, and cell culture techniques have made possible the study of reactions and interactions of well defined cell populations in response to viral stimuli. With the identification, by Isaacs and Lindenmann in 1958, of a protein substance produced in response to viral infections and capable of inhibiting virus multiplication and virus-induced cell damage (32), a great surge of interest was aroused in the biochemistry of cellular responses to viruses. Of special interest was the observation that this protein, now called interferon,

acted on the susceptible cell rather than on the virus itself, as does antibody.

Concomitantly, immunologists were becoming increasingly aware of the versatility of the lymphocyte which, they found, could respond to various stimuli by the production of proteins capable of causing striking changes in other cells (3). The old distinction between cellular and humoral immunity and the histologic changes accompanying each could now be viewed as resulting from the qualitatively different products produced by cells in response to exogenous stimuli. The cellular products in delayed hypersensitivity have their primary action on other cells, whereas the cellular products in humoral immunity have their primary action on the stimulus itself.

While the importance of interferon in recovery from viral infections has justifiably gained wide and rapid acceptance, the role of the more conventional immune responses has been largely neglected or dismissed (2, 29). Recent reviews of the subject have considered and contrasted the relative roles of interferon, antibody, and delayed hypersensitivity as if they were entirely separable entities. Reflecting the experimental questions asked by individual investigators, these reviews have emphasized the differences in the three responses and, from these differences, have extrapolated to differences in significance.

This thesis presents experimental evidence linking interferon production to delayed hypersensitivity and reinterprets some of the relevant preexisting data. It emphasizes the immunologic rather than the non-immunologic aspects of interferon production, and aims toward a more unified and comprehensive mechanism for recovery from viral infections.

A. INTERFERON

1. General Properties

Available evidence indicates that interferon is a protein with the ability to inhibit viral multiplication intracellularly (37). It has a molecular weight of 25,000-100,000, is stable over the pH range 1-10, and slowly loses titer at 37°C. but not at -20°C. Its anti-viral effect is nonspecific; to varying degrees it will inhibit multiplication of virtually all animal viruses. Its effectiveness is species specific; its anti-viral effect is best or solely demonstrated in cells or animals of the species from which it was derived.

2. Methods of Induction

Interferon can be isolated from the serum or organs of many different animals infected with RNA or DNA viruses. The *in vitro* production of interferon has been observed in cell lines from species as diverse as fish and man (7). Differences in the ability of different cells to produce interferon have remained unexplained. Likewise, it is not known why some viruses are better inducers of interferon than others. In most mouse cells, arboviruses have been good inducers of interferon.

The mechanism of interferon induction and the characteristics of the interferon produced seem to vary according to the nature of the inducing agent. One class of inducers is the viruses; the currently accepted hypothesis is that they derepress a host cell gene which codes for interferon (15). Endotoxin is the prototype of another class of inducers which are thought to cause the release of "preformed" interferon rather than initiate its *de novo* synthesis (30, 31).

It has been shown that peak titers of endotoxin-induced interferon are reached much more rapidly than peak titers of virus induced interferon (31). Also, in vivo concentrations of actinomycin D sufficient to inhibit virus-induced interferon production do not inhibit endotoxin-induced interferon production (31).

The validity of using these observations as the basis for making a mechanistic distinction between classes of inducers may be questioned. Many attempts to induce interferon with endotoxin in vitro have failed, and the endotoxin experiments which use inhibitors of RNA and protein synthesis have been done in vivo. Recently, Smith and Wagner reported in vitro endotoxin-induced interferon production in purified rabbit macrophages (46). They found that induction by this method was inhibited by actinomycin D. Sauter and Gifford measured in vivo levels of interferon and a lysosomal enzyme following intravenous administration of endotoxin and found that both reached maximum titers at two hours (43). They concluded that interferon may be released from lysosomes. Another interpretation is that interferon increases cell membrane permeability to proteins, thereby allowing interferon to escape more rapidly than it does with virus-inducers.

Endotoxin-induced interferon has also been observed to have different physical characteristics from virus-induced interferon. It is reported to have a greater molecular weight (30) and to be less heat and acid stable than virus-induced interferon from the same animals (31). Until interferon can be purified, it is difficult to know the significance of these observations.

Other aspects of endotoxin-induced interferon must be considered by any investigator working with culture media that may contain pyrogen. Two

investigators have demonstrated *in vitro* interferon production induced by endotoxin; in one case, anti-endotoxin antibody enhanced the response (46, 6). Tolerance, or the inability of animals or cells to make interferon for a variable period after injection of virus or endotoxin, may prove to have *in vitro* significance. For instance, some cells are unable to produce interferon in response to viruses if they are cultured for several hours before challenge (5, 53). Conceivably, this phenomenon could be due to tolerance induced by endotoxin present in the media.

3. Mechanism of Action

The mechanism of action of interferon is unknown. Interferon does not directly inactivate virus particles or infectious nucleic acid, nor does it affect virus adsorption, penetration, or uncoating. It does not appear to inhibit host cell protein synthesis or the activity of specific viral enzymes (45).

Studies with antimetabolites have shown that host cell DNA-dependent RNA synthesis and protein synthesis are necessary for interferon action. The hypothesis that interferon acts as a derepressor for the synthesis of another cellular protein, which may act in conjunction with interferon or may be the anti-viral molecule itself, is consistent with available experimental data. Recent evidence, reviewed by Vilček, has focused attention on the ribosome as the site of anti-viral action (50).

As with interferon induction, variability in the responsiveness of different cells or viruses to the action of interferon is a frequently observed but poorly understood phenomenon. Vesicular stomatitis virus and mouse L cells have been widely used as an assay system for mouse interferon.

Even with this system, however, different sensitivities have been reported for different clones of cells and mutants of the virus (36).

B. INTERFERON PRODUCTION BY LYMPHOCYTES AND MACROPHAGES

Lymphocytes and macrophages have been shown to produce interferon *in vitro* and indirect evidence implies that they are also important *in vivo* sources of interferon (13, 28, 35, 46, 53).

The most convincing *in vivo* evidence has been presented by DeMaeyer *et al*, who irradiated C_3H/He mice with 1000 rads, restored their bone marrow with Wistar rat marrow cells, and three weeks later challenged them with Newcastle Disease Virus (13). Whereas unrestored irradiated animals produced no detectable circulating interferon in response to the challenge, animals restored with rat cells produced interferon proportional to the rate of marrow repopulation and having the specificity of rat interferon.

In vitro studies designed to elucidate which of the bone marrow-derived cells produce interferon show that both macrophages and lymphocytes produce interferon. However, the details of the experiments caution against generalization. Rabbit macrophages produce interferon in response to viruses and purified endotoxin (46), whereas mouse macrophages respond to viruses but not endotoxin (48). It is not known whether this represents a relative or absolute species insensitivity to endotoxin. Studies with lymphocytes indicate that these cells make interferon in response to viruses, phytohemagglutinin, pokeweek mitogen, and streptolysin O (20, 28, 53). These experiments with lymphocytes all used human blood cells and, although attempts were made in two cases to purify the cells, the reports lacked a critical evaluation of the purification procedures. Because of this, some

doubt must remain as to which cell type in those preparations produced the interferon, but the burden of proof now seems to fall on those who would maintain that lymphocytes do not produce interferon.

The methods of collection and culture of cells can influence interferon production. Wheelock observed that if his purified lymphocytes were cultured for more than two hours before challenge with NDV they lost their ability to produce interferon (53). This was not, however, observed if PHA was the inducer. Smith and Wagner found that all of their culture media contained pyrogen and that cultures of uninfected rabbit macrophages produced low titers of interferon (46). Also, macrophages from peritoneal cavities stimulated with glass beads 72 hours before collection produced far more baseline interferon than macrophages from unstimulated peritoneal exudate. The authors suggest that nonspecific mobilization of macrophages can partially activate them to produce interferon.

C. DELAYED HYPERSENSITIVITY

1. In Vivo

Delayed hypersensitivity is the capacity of animals to manifest characteristic pathological changes in response to antigenic stimuli. The histologic features of this reaction to antigen are perivascular and tissue accumulations of lymphocytes and macrophages followed by widely varying degrees of tissue necrosis, fibrinoid necrosis of blood vessels, and accumulation of polymorphonuclear leucocytes (51). The chief operational characteristics of delayed hypersensitivity are its immunologic specificity and its requirement for specific lymphoid cells rather than humoral antibody.

On the basis of similar histologic features and the above mentioned operational requirements, such seemingly diverse entities as tuberculin skin sensitivity, contact allergy, and homograft rejection can all be termed delayed hypersensitivity reactions.

2. In Vitro

Studies of lymphocytes in vitro have greatly aided the understanding of the mechanism whereby these cells effect the pathological changes observed. In the presence of antigen, lymphocytes from animals specifically sensitized to that antigen have been shown to release several biologically active molecules (all tentatively identified as proteins). These include a factor which inhibits macrophage migration (4, 12), a factor which is cytotoxic for a variety of cells (42, 25), a factor which is chemotactic for macrophages (52), a factor which induces blast transformation in lymphocytes (17), and a factor which transfers delayed skin reactivity (34). Normal lymphocytes will respond to mitogens by producing some of the above factors and, in addition, interferon (3). Why interferon is found in consort with these other proteins is unknown. Furthermore, the possibility exists that a single protein may possess the properties of several of the above mentioned factors.

3. In Mice

Most demonstrations of delayed hypersensitivity in mice have been indirect (increased susceptibility to endotoxin shock) (26) or unconvincing (11, 1), except when replicating antigens such as live Mycobacterium hominis have been used (14). Kantor recently reviewed and clarified the problem by testing 25 mouse species for delayed hypersensitivity

to heat killed Mycobacterium (33). He found marked species variability to footpad injections of PPD and showed that BALB/c and C_3H mice were the best reactors. Gross measurement of the feet correlated well with the severity of the histologic lesions. The delayed hypersensitivity could be transferred to normal mice with peritoneal exudate cells but not with spleen or lymph node cells.

4. In Virus Infections

The histologic changes seen in virus infections are frequently indistinguishable from those seen in delayed hypersensitivity reactions. Whether these changes represent an immunologic reaction to viral antigens or are the nonspecific consequence of direct cell damage caused by the virus is unknown. There is some experimental evidence, as well as interpretations of "experiments of nature," that suggest a role for delayed hypersensitivity in virus infections.

People with agammaglobulinemia in whom there is no deficiency in cellular or delayed immunity, as evidenced by their ability to react in the usual way to skin grafts or skin tests for delayed hypersensitivity, have no difficulty recovering from most viral infections. In contrast, people with congenital deficiencies in their ability to develop delayed reactions often die in childhood from viral illnesses (44).

Experimentally, peritoneal cells, but not serum, from animals infected with fibroma virus confer partial resistance to fibroma in other animals (22). The macrophage migration inhibition experiment, described by George and Vaughn (21) and interpreted as an in vitro manifestation of delayed hypersensitivity, also works using cells from mice immunized with mumps and

influenza viruses (16). Newborn rabbits, rendered tolerant to vaccinia by the injection of heat killed virus, developed progressively lethal vaccinia when challenged several days later with live virus; none of the control animals died (18). Interestingly, only a minimal local lesion was found in the tolerant animals and the investigators cautiously suggested that the skin lesion of primary vaccinia, as well as the prevention of generalized vaccinia in newborn rabbits, was due to delayed-type hypersensitivity.

Using a different approach, others have shown that guinea pigs made immunologically unresponsive by x-irradiation and treatment with methotrexate recovered from vaccinia infections as rapidly as controls, but were subsequently unable to give delayed skin reactions to heat-killed vaccinia (19). Furthermore, the amount of interferon found in homogenates of primary skin lesions was equal in immunologically paralyzed and in control animals. These results were interpreted as showing that interferon was responsible for the recovery from viral infections and that delayed hypersensitivity and antibody played little, if any, role. This work would have been far more convincing if it had demonstrated that the "immunologically unresponsive" animals also failed to react to more typical delayed hypersensitivity-evoking stimuli.

5. Acquired Cellular Resistance

Animals infected with intracellularly replicating bacteria develop both an immunologically specific and nonspecific ability to resist reinfection (38). Macrophages from such animals have an increased capacity to destroy ingested microorganisms, even in the absence of conventional antibody. Macrophages are thus thought to be the effector cell for the

in vivo phenomenon called *acquired cellular resistance*. Such resistance is believed to be the result of nonspecific activation of macrophages secondary to specific lymphocyte-antigen interaction. It has been observed with bacteria, fungi, and protozoa and correlates well with increased macrophage production of lysosomal enzymes (38). Acquired cellular resistance has been used to explain the fibroma virus experiment mentioned above as well as other demonstrations of decreased virus replication after reinfection (47). It adds little, however, to the understanding of the mechanisms involved in these virus experiments unless it can be distinguished from or correlated with known immune reactions and/or interferon.

Animals displaying acquired cellular resistance also commonly have delayed hypersensitivity to the infecting organism. The relationship between these two altered states of reactivity is unknown, but two possibilities have been suggested (39): [1] sensitized lymphocytes react with antigen and produce a pharmacologically active substance which in turn causes changes in macrophages; [2] sensitized lymphocytes react with antigen and produce a cytophilic antibody which, when combined with antigen, causes changes in macrophages.

D. EFFECT OF IMMUNITY ON INTERFERON PRODUCTION

Animals making interferon in response to a virus develop immunity (cellular or humoral or both) to that virus. How immunity affects or relates to interferon production was the subject of experiments reported by Glasgow (23). Peritoneal cells from CD-1 mice immunized against Chikungunya virus (CV) were challenged *in vitro* with live CV. Culture fluids assayed for interferon showed that cells from immune animals produced two to ten

times more interferon than cells from control animals. This increase was specific for CV; other viruses did not produce the effect in CV immune cells. It was also shown that the rate of adsorption of CV was equal in control and CV immune cells; that virus neutralized with antibody resulted in a poor but equal response in both normal and immune cells; and that normal cells exposed to anti-CV antibody and then washed produced nearly as much interferon in response to CV as cells not exposed to antibody. Glasgow's conclusion was that the enhanced interferon production by immune cells provided a mechanism whereby cellular immunity could influence viral infections.

* * *

The work to be described in this thesis was undertaken to confirm and extend Glasgow's observations. Of special interest were the relative roles of macrophages and lymphocytes in the enhanced response observed with crude peritoneal exudate cells. Experiments were designed to test two theoretical interpretations of Glasgow's results:

1. If increased interferon production is analogous to acquired cellular resistance, macrophages should be the important effector cell. It might also be expected that lymphocytes are important in making the reaction immunologically specific.

2. If the increased interferon production is a manifestation of a delayed hypersensitivity reaction, then other <u>in vitro</u> examples of delayed hypersensitivity reactions should yield the same result.

MATERIALS AND METHODS

A. MICE

Random-bred six week old male Swiss ICR mice, from a colony maintained by the Division of Animal Care at Yale, were used for all experiments with viruses and for the initial experiments with tuberculin-sensitized animals. Inbred six week old male BALB/c mice, obtained from Jackson Farms, Bar Harbor, Maine, were used for the definitive studies with tuberculin-sensitized animals.

B. CONTINUOUS LINE CELL CULTURE

L cells (clone 929), an established line of mouse fibroblasts, were a gift from Dr. Hilton B. Levy. Cells were grown in growth medium (see Appendix) in 250 ml. screw-top plastic flasks (Falcon Plastics) which were gassed with a mixture of 5% CO_2:95% air. Cells were transferred by decanting the growth medium, washing the cell sheet with Saline A, and incubating for five minutes at 37°C. with 0.5 ml. of a trypsin (0.125%): versene (1:10,000) mixture. Cells were split and transferred 1-2 times per week, depending on the need for them. Attempts to isolate possible mycoplasma contaminants from these cells and culture media were negative.

C. VIRUSES

All viruses were obtained from the W.H.O. Arbovirus Reference Laboratory of the Yale Arbovirus Research Unit. Two pools of Chikungunya virus (CV, 175th baby mouse brain passage) were prepared by intracerebral inoculation of three day old mice with 0.02 ml. of the 10% virus

preparation reconstituted with PBS. Two days after infection, the brains of dying mice were aseptically removed with scissors and forceps, made into a 10% suspension in PBS by repeated passage through a 20 guage needle, and centrifuged for 20 minutes at 10,000 r.p.m. (12,350 g) in a Sorvall RC-2 refrigerated centrifuge. Aliquots of the supernatant were placed in ampoules, shell-frozen in dry ice-ethanol, and vacuum desiccated for six hours at 1m of Hg. The ampoules were then filled with N_2, sealed, and stored at -70°C. in a Revco freezer. These preparations maintained their titers for at least three months. (E.G., Pool 1 had an intracerebral titer of $LD_{50}=10^{-7.6}-10^{-8.0}/0.02$ ml. in suckling mice.)

Vesicular Stomatitis Virus (VSV), Indiana strain, from the eighth baby mouse brain passage, was prepared in pools in a manner similar to that for CV except that it was suspended in PBS + 0.75% bovine albumin.

West Nile virus (WN) was from the eleventh baby mouse brain passage.

D. METHODS OF SENSITIZATION

1. Virus

Groups of 20-30 six week old Swiss ICR mice were immunized by three weekly intraperitoneal injections of 0.1 ml. of live Chickungunya virus, rehydrated from the lyophilized form to a 10^{-1} dilution with sterile PBS. An equal number of animals injected intraperitoneally with 0.1 ml. of a 10% suspension of baby mouse brain (BMB) served as controls. Three to twelve weeks after the last injection, the animals were sacrificed for *in vitro* testing of their peritoneal cells.

One group of animals was immunized as above except that the virus

suspension was emulsified with an equal volume of Freund's incomplete adjuvant (85% Bayol F, 15% Arlacel A).

Another group of animals was immunized by 0.033 ml. of live CV (10^{-1}) given in each of three footpads. This group received two such injections and was sacrificed three weeks after the last injection.

An average of one animal per group died before sacrifice.

2. Mycobacterium Tuberculosis (MT)

Mycobacterium tuberculosis hominis, strains C, DT, and PN, heat killed, washed, and dried on 9/24/58, were obtained from Dr. Byron Waksman. Both Swiss ICR and BALB/c mice were immunized with MT.

Groups of 40-50 six week old mice were anesthetized with ether and injected in each of three footpads with 0.033 ml. of MT suspended in Bayol F (1 mg./ml.). Each animal thus received 100µg. of MT. Control animals were either sacrificed for in vitro testing of their peritoneal cells or were footpad tested with PPD.

E. FOOTPAD TESTING

Animals were anesthetized with ether and tested in the footpad which had not been used for immunization ten days previously. The test dose was 25 µg. PPD (Parke, Davis & Co.) in 0.05 ml. PPD diluent. Twenty-four hours later, the animals were killed and the largest dorsal-ventral diameter of the footpad was measured with a vernier caliper.

F. IN VITRO CULTURE OF MOUSE PERITONEAL CELLS

1. Crude Peritoneal Exudate

Mice were rapidly killed with ether. The abdominal skin was washed with 70% ethanol and reflected. All further manipulations were done aseptically. With a Pasteur pipette, 1 1/2 - 2 ml. of cold (4°C.) peritoneal exudate harvesting medium (see Appendix) was introduced into the peritoneal cavity. Ten minutes later the fluid was withdrawn, using a Pasteur pipette, and centrifuged for five minutes at 900 r.p.m. (300 g) in a refrigerated International Centrifuge. The resulting supernatant was carefully and thoroughly removed by aspiration, and the cells were resuspended in peritoneal exudate maintenance medium (see Appendix) by gentle pipetting.

The cells were counted and distributed to 75 ml. (25 mm^2) plastic screw-top flasks (Falcon Plastics) so that each flask contained $5x10^6$ cells in a final volume of 4 ml. maintenance medium. Flasks were gassed with 5% CO_2:95% air, tightly capped, and incubated at 37°C. Each mouse yielded $2-4x10^6$ cells.

2. Purified Macrophage Preparation

Crude peritoneal exudate was prepared as above. 10^7 cells were placed in each flask and, after incubation for one hour at 37°C., non-adherent cells were removed by gentle agitation and aspiration. Adherent cells were washed two times with maintenance medium. Approximately $5-6x10^6$ cells remained adherent after washing and these were predominantly macrophages (see below).

3. Purified Lymphocyte Preparation

The non-adherent cells from the first aspirate of the crude peritoneal exudate preparation which had been allowed to incubate for one hour at 37°C. were predominantly lymphocytes. They could be further purified by another one hour incubation at 37°C. Approximately 3-4x10^6 non-adherent cells could be recovered after the second adsorption, and these were predominantly lymphocytes (see below).

G. CELL STAINING AND ENUMERATION

1. Counting

Cells were diluted in Turk's solution (3% acetic acid, 1% crystal violet) and counted in a Beckman hemocytometer.

2. Giemsa

Using a Giemsa stain of mouse peritoneal cells prepared in a manner similar to that described above, Cohn reported that 50-60% of the crude exudate cells were macrophages while the rest were lymphocytes (9). In trying to duplicate that observation using a standard Giemsa stain, it was found that approximately 50% of the cells were easily identified as macrophages and 30% as lymphocytes, while the remaining 20% were either poorly stained, smudged or otherwise indistinguishable. An occasional mast cell (<1%) and a very occasional polymorphonuclear neutrophile (<<1%) were seen.

3. Carbon Ingestion

A physiologic differentiation of the two types of cells was therefore

attempted. Crude peritoneal exudate and purified preparations of "macrophages" and "lymphocytes" were prepared in Leighton tubes with coverslips cut from the plastic bottoms of Falcon flasks. Each preparation was then incubated for one hour at 37°C. with carbon (Pelikan $C^{11/1431}$) diluted 1:10,000. Microscopic examination showed that 63% of the crude exudate cells, 97% of the "macrophage" preparation, and 7% of the "lymphocyte" preparation ingested carbon particles (>5/cell). It should be noted that these "purified" cell populations did not have the extra adsorption and washing steps used subsequently in preparing cells for experiments.

4. Viability

Cell viability was determined using the trypan blue exclusion technique. Cells were exposed to a 1:5 dilution of 0.4% trypan blue (Allied Chemical) for five minutes and examined microscopically. In preparations of crude peritoneal exudate, macrophages, and lymphocytes, an average of 99%, 93% and 96% of the cells, respectively, excluded the dye.

H. INTERFERON PRODUCTION IN VITRO

Cells to be tested for their ability to produce interferon were prepared in plastic flasks as described above. In virus experiments, duplicate cultures of cells from CV immune animals and BMB immune animals received 0.5 ml. CV (10^{-1} dilution of the mouse brain preparation in maintenance medium). In most experiments CV immune cells, to which 0.5 ml. 10% BMB had been added, served as a control. After preparations were gassed and incubated 24 hours at 37°C., the culture medium was assayed for interferon.

In experiments with tuberculin sensitized cells, 25 µg./ml. PPD was added to the culture medium, the flasks were gassed, and then incubated for 24 hours at 37°C. Control preparations are described in Results.

I. INTERFERON ASSAY

1. Preparation of Samples

Preparations to be assayed for interferon were clarified by centrifugation for 20 minutes at 10,000 r.p.m. (12, 350 g) in the refrigerated Sorvall. In the experiments where PPD was the interferon inducer, the clarified supernatant was assayed directly.

When live virus was used as the interferon inducer, it had to be killed before the preparations could be assayed. This was achieved by aseptically pipetting the clarified preparation into 5/8 inch diameter dialysis tubing (Arthur H. Thomas Company) which had been knotted at one end and autoclaved in distilled water for 15 minutes. The other end of the tubing was then sealed with another knot and suspended by a string from the lip of an Erlenmeyer flask. The fluid in the tubing was dialyzed for 24 hours at 4°C. against 20-30 volumes (as compared to the total volume of the preparations) of HCl, pH 2.0, and then neutralized by a six hour dialysis against ten volumes of Saline A. All preparations were stored at -40°C.

A laboratory interferon standard was prepared from the brains of five week old male mice which had been intracerebrally infected with 0.1 ml. West Nile virus diluted 10^{-4}. Three days after infection a 20% brain suspension in PBS was made from those animals and treated as above for virus-induced interferon preparations. The West Nile preparation maintained

a constant titer for more than six months when stored at -40°C.

2. The Assay

Interferon activity was assayed by the plaque reduction method using L cells and Vesicular Stomatitis Virus. Interferon preparations were diluted by serial 2- or 4-fold dilutions in diluent (see Appendix) at 37°C. VSV was diluted with cold diluent so that 0.2 ml. of the final dilution would contain approximately 30-50 plaque forming units. 1.5×10^6 L cells in 8-10 ml. of growth medium were pipetted into six cm. multiwell plastic plates (Linbro FB6-TC) and incubated at 37°C. in a humidified 5% CO_2 atmosphere. In two to three days, when the cell sheet was a confluent monolayer, the growth medium was removed and two ml. of the interferon preparation was added and allowed to incubate at 37°C. for 3-20 hours. The incubation time in most experiments was six hours. The interferon preparations were then removed, the cell sheet was washed once with five ml. of warm PBS, and 0.2 ml. VSV was dropped onto the cells and incubated one hour at 37°C. The cell sheets were then overlaid with five ml. of the first agar overlay (see Appendix) and returned, inverted, to the incubator. The second agar overlay (see Appendix) was applied at 24-40 hours and the plaques were counted at 40-48 hours.

3. Variability

The variability in the triplicate platings of the sample dilutions is somewhat greater than that usually acceptable in standard virus plaquing techniques. Similar variability is present, however, in the early interferon literature in which actual plaque counts were reported (19). In retrospect, the most likely source for the variability in the plaquing reported here is

in the method used to prepare the cell sheets. Aliquots of cells were pipetted in 0.5 ml. amounts into the dishes containing growth medium. A better method would have been to dilute the cells in the total volume of growth medium, thereby allowing larger volumes to be pipetted. In spite of the variability, *t* tests reveal that the differences between some of the comparable samples are significant, while the differences between others are not.

RESULTS

A. INTERFERON PRODUCTION BY CELLS FROM TUBERCULIN SENSITIZED ANIMALS

Preliminary studies with peritoneal cells from ICR mice immunized with MT showed interferon production *in vitro* proportional to the amount of PPD added. Since PPD in concentrations greater than 50 µg./ml. is known to be cytotoxic, all further experiments used PPD at a concentration of 25 µg./ml. The results of two early experiments, although not statistically significant, suggested that the cells from immunized animals produced more interferon in the presence than in the absence of PPD, and that cells from control animals did not respond in that manner.

Kantor has shown that different strains of mice vary greatly in their ability to exhibit tuberculin sensitivity, and that BALB/c mice are among the best responders (33). BALB/c mice were, therefore, used in the rest of the experiments to be described, and their delayed hypersensitivity to PPD was assessed by footpad testing. The results of such a test are shown in Table 1.

Medium from the incubation of crude peritoneal exudate cells with and without PPD was assayed for interferon. The results, summarized in Table 2 and Figures 1 and 2, show that cells from immune animals produced more interferon (as evidenced by plaque inhibition) in the presence of PPD than in its absence, whereas cells from control animals show no difference in interferon production. Table 2 also shows the results of a significance test done on these differences. Since it had been found that PPD affected the titration by increasing the number of plaques formed by a given amount of VSV, it was necessary to make the raw data comparable before calculating

the t values. This was done by multiplying the number of plaques formed in PPD-containing samples by the factor which would make the expected number of VSV plaques formed in the presence of PPD equal to the number formed by VSV in the absence of PPD.

As part of the last described experiment, purified macrophages and lymphocytes from the same group of animals were tested for their ability to produce interferon. It appeared that each type of cell produced some interferon, but that there was no difference in the responsiveness to PPD whether the cells came from sensitized animals or controls. This was also true for lymphocyte preparations cultured with or without PPD for 96 hours.

Since each cell type alone did not appear capable of producing the response observed in the crude exudate preparations, the following experiment was done. Macrophages and lymphocytes, prepared in the usual manner, were recombined: sensitized lymphocytes with sensitized macrophages, sensitized lymphocytes with normal macrophages, normal lymphocytes with sensitized macrophages, and normal lymphocytes with normal macrophages. These were then incubated with or without PPD and the medium was assayed for interferon. The results, summarized in Table 3, suggest that, in the presence of PPD, sensitized lymphocytes with either tuberculin-sensitized or normal macrophages produce more interferon than these cells in the absence of PPD. The results of the footpad test done on litter mates of the animals used in the experiment are shown in Table 1.

B. INTERFERON PRODUCTION BY CELLS FROM CV-IMMUNIZED ANIMALS

The major portion of the experiments performed during the course of this study were done before the PPD experiments and were attempts to confirm

and extend the work reported by Glasgow with CV immune cells. In spite of the use of a variety of immunization routes, cell collection techniques, and cell culture conditions, none of the experiments resulted in evidence that cells from animals immunized with CV produced more interferon when exposed to CV *in vitro* than did cells from non-immune animals. Experimental protocols tried were: immunization by the intraperitoneal or intradermal routes with or without an oil adjuvant; collection of uninduced peritoneal exudates or exudates induced for 24 hours with trypticase soy broth; culture with either heat-inactivated fetal calf serum or heat-inactivated calf serum; and culture with or without antibiotics. Except where noted, the few experiments to be described followed the protocol as outlined in *Materials and Methods*.

Early experiments (Table 4) showed that only very high multiplicities of virus induced interferon production *in vitro*, whether the cells came from animals immunized with CV or control animals which had received intraperitoneal injections of baby mouse brain (BMB).

A typical result from an experiment in which no antibiotics were used in the interferon preparations is seen in Table 5, which also shows the transformation of raw data into averages and percentages. The results fail to show any difference in plaque reduction caused by medium from CV cells exposed to CV when compared to medium from BMB cells exposed to CV.

Of interest in these experiments was the observation that the purified preparations of macrophages and lymphocytes, obtained either from CV immune or control animals, and cultured *in vitro* with CV, produced a substance which depressed VSV plaque formation (Table 6). It should be noted that culture fluids from uninfected cells greatly increased the number of plaques

formed by a standard amount of VSV. Using the number of plaques formed by VSV on cells treated with these uninfected culture media as the expected or 100% number of plaques, probit plots can be constructed which yield 50% plaque-depressing dilution of the preparations (Figure 3).

DISCUSSION

The experimental work presented in this thesis provides evidence that peritoneal cells from animals with delayed hypersensitivity to PPD produce an enhanced interferon response when exposed to PPD *in vitro*, whereas cells from control animals do not. Cell separation and recombination experiments were done to ascertain the roles of macrophages and lymphocytes in producing this response. Similar experiments using Chikungunya virus as immunogen and interferon inducer failed to demonstrate any difference between cells from immune and control animals.

Absent from the execution, but not the design, of these experiments was a demonstration that the viral interference which was repeatedly observed could be attributed to a substance whose biological and chemical properties allow it to be called an interferon. Therefore, *interferon* as used in discussing the work described here refers to the ability of certain culture media to inhibit VSV plaque formation on L cells when compared to an appropriate control.

What were the appropriate controls? In the experiments reported by Glasgow, cells from immunized animals were compared with cells from non-immunized animals. Theoretically, at least, such a comparison is probably not valid. Immunization with a replicating antigen (BCG) has been shown to alter the relative proportions of the different cell types in guinea pig peritoneal exudate (40). Nonspecific "activation" of macrophages in response to polyanions as described by Cohn (10) may also occur *in vivo*, and then one must wonder how long such cells remain "activated," and if such nonspecific activation could include interferon production as suggested by Smith and Wagner (46).

To allow for these possibilities, control cells for the virus experiments were from animals receiving injections of baby mouse brain. The primary aim of the virus experiments was to repeat Glasgow's observation and, had this been done, a more ideal control would have been to immunize animals with two different viruses and show that the enhanced *in vitro* interferon response was specific for the immunizing virus.

Control animals in the tuberculin experiments received injections of Bayol F; however, a different heat-killed bacterium like Brucella or Listeria would have been a better control. The theoretical objections were somewhat circumvented in these experiments by looking for differences in the same cells exposed to different stimuli, rather than for differences in different cells.

One difficulty with the latter approach was that it became obvious that titration controls were crucial. The effect of PPD on VSV plaque formation had to be taken into account, as described above. In the absence of more complete evidence that the plaquing inhibitor observed was, in fact, interferon, the possibility exists that the inhibitor acted indirectly by preventing this plaquing enhancement caused by PPD.

The results of the PPD experiments suggest that interferon is produced when tuberculin-sensitized cells are cultured in a manner similar to that used in demonstrations of *in vitro* manifestations of delayed hypersensitivity (MIF, CF, etc.). The report by Green *et al*, showing interferon production by lymphocytes from PPD-positive humans, cannot be compared with MIF or CF production since these authors cultured their cells for four to ten days (27).

It was of interest to know whether a single cell type could produce the enhanced response or whether an interaction between lymphocytes and

macrophages was necessary. The recombination experiment showed that the sensitized lymphocyte is the key cell but says nothing about the necessity for macrophages. One experiment using purified cell populations suggested that sensitized lymphocytes alone cannot produce the observed response. Unfortunately, great variability in the titration controls for that particular experiment prevented meaningful comparisons, and no conclusion can be made from that experiment. Furthermore, the "purified" cell populations are still heterogeneous enough to make one hesitate to draw any conclusions about specific cells acting alone. For example, consider the small amounts of interferon produced in the virus experiments by the lymphocyte preparation (Figure 3). Does it come from low but detectable levels of production by the lymphocytes or from the 5% contaminating macrophages which are good interferon producers? This type of problem could be approached by mixing different proportions of "macrophages" and "lymphocytes."

With regard to controls for the virus experiments, it was found that acidified calf serum increased the number of plaques formed by a standard amount of VSV. However, all culture media contained serum and this factor should not, therefore, have influenced comparison of preparations. On the other hand, the influence of acidified CV on VSV plaquing was not determined and, in retrospect, this would have been a desirable control.

The failure of the virus experiments described here to confirm the work reported by Glasgow is rather typical of interferon work. Small differences in virus strain, cell clone, or animal species often greatly influence interferon production and assay. The animals and cells used in the present research may differ slightly from those used by Glasgow;

neither work serologically confirmed the identity of the virus used. Glasgow, himself, has been unable to repeat his experiment using different viruses, but reports that a Japanese group successfully repeated the experiment using a virus with which he was unsuccessful (24). Subrahmanyan and Mims reported that they were unable to confirm Glasgow's experiment, but their experimental design was significantly different from his (48). Using an assay system in which the interferon-producing ability of individual spleen cells could be assayed, Osborn reported preliminary results which suggested that cells from immune animals were better producers of interferon when challenged with the homologous virus than were non-immune cells (41).

Although the experimental results described in this thesis do not allow generalizations concerning delayed hypersensitivity, interferon production, and recovery from viral infections, they are suggestive enough to warrant a reevaluation of some heretofore puzzling experimental results. Stineberger and Rights found that vaccinia replicated far less well in freshly explanted spleen cells from vaccinated than from nonvaccinated rabbits (47). The difference was specific for vaccinia, was not observed with kidney cells, and could not be demonstrated if the spleen cells were first cultured for seven days. Flick and Pincus showed that rabbits tolerant to vaccinia develop generalized fatal vaccinia and do not produce the usual local vaccinia lesions (18). They suggested that delayed hypersensitivity was important in lesion formation and in protection against generalized vaccinia. Is it possible that localized delayed hypersensitivity reactions induce interferon production which is instrumental in localizing and inhibiting the virus?

Turk _et al_ sensitized guinea pigs with ultraviolet inactivated

vaccinia and challenged them with live vaccinia at a time (five days) when there was no circulating antibody to vaccinia (49). The skin test sites were then excised and titered for vaccinia. At 48, but not 24 hours after challenge, there was a ten fold greater amount of virus in non-immunized animals as compared to immunized animals. Animals sensitized with Mycobacterium tuberculosis and challenged with a mixture of PPD and vaccinia showed a "small but significant" decrease in vaccinia replication as compared to MT sensitized animals challenged with vaccinia but no PPD. Because the immunologically nonspecific or unrelated tuberculin reaction inhibited vaccinia multiplication nearly as well as the postulated specific reaction in vaccinated animals, the authors concluded that delayed hypersensitivity does not play an important part in the resolution of vaccinia infections. Had these authors known that delayed hypersensitivity reactions to PPD could result in interferon production, they might have interpreted their results as showing that delayed hypersensitivity to viruses inhibits virus multiplication and that this effect is mediated by increased interferon production at the reaction site.

SUMMARY

The importance of interferon in the recovery from viral infections cannot be denied. Yet clinical observations, as well as some experimental data, suggest that the ability to develop delayed hypersensitivity reactions may also play a role in the normal recovery process. The mechanisms whereby interferon and delayed hypersensitivity reactions influence the outcome of viral infections may be entirely distinct, or they may be related. Experimental data reported in this thesis provide a link between the two and suggest a mechanism that would explain the role of delayed hypersensitivity. The data presented show that peritoneal cells from animals exhibiting delayed hypersensitivity to PPD produce more interferon when cultured *in vitro* with PPD than do cells from nonsensitized animals. This result appears to require the presence of both lymphocytes and macrophages. Cell separation and recombination experiments show that increased interferon production occurs only when the lymphocytes are obtained from sensitized animals, but that the macrophages may be obtained from either sensitized or nonsensitized animals. Attempts to show enhanced interferon production by cells from virus-sensitized animals exposed to the sensitizing virus were unsuccessful. Several experiments in the literature are reappraised in view of the possibility that interferon production as a manifestation of delayed hypersensitivity can influence viral infections.

Table 1

FOOTPAD TEST

Sensitizing Agent	Challenge	Largest Dorsal-Ventral Diameter (mm.)	Average Dorsal-Ventral Diameter	% Increase Over Diluent Control
MT*	PPD	4.1		
		4.0		
		3.5		
		3.3		
		2.8	3.54	37.4
MT	Diluent	2.8		
		2.7		
		2.6		
		2.5		
		2.5	2.62	
BF**	PPD	2.9		
		2.8		
		2.7		
		2.7		
		2.7	2.76	6.2
BF	Diluent	2.7		
		2.6		
		2.6		
		2.6		
		2.5	2.60	

Footpad measurements were made 24 hours after challenge and 11 days after sensitization.

*Myobacterium tuberculosis

**Bayol F

Table 2

EFFECT OF PPD ON INTERFERON PRODUCTION BY CRUDE PERITONEAL CELLS FROM TUBERCULIN SENSITIZED AND CONTROL ANIMALS

In Vivo Sensitizing Agent	In Vitro Challenge	Dilution of Culture Supernatant 1:5 Avg. No. of Plaques*	p**	1:10 Avg. No. of Plaques	p	1:20 Avg. No. of Plaques	p
MT	PPD	52 ± 3.0	<0.01	70 ± 5.7	<0.02	84 ± 2.5	<0.001
MT	Diluent	81 ± 9.4		95 ± 8.3		105 ± 5.0	
BF	PPD	77 ± 4.2	<0.8	82 ± 2.3	<0.3	97 ± 4.5	<0.5
BF	Diluent	74 ± 7.8		89 ± 9.3		105 ± 7.8	

Cells from animals sensitized ten days before 24 hour in vitro challenge. Plaques counted 48 hours after L cells had been treated with supernatants and infected with VSV.

MT - Mycobacterium tuberculosis
BF - Bayol F

*From 9 plates for each dilution; triplicate determinations of three identical samples ± standard error of the mean.

**From Student's t test, where $t = \dfrac{\bar{x}_1 - \bar{x}_2}{\left[\dfrac{(x_1-\bar{x}_1)^2 + (x_2-\bar{x}_2)^2}{n_1+n_2-2}\left(\dfrac{1}{n_1}+\dfrac{1}{n_2}\right)\right]^{\frac{1}{2}}}$

(Raw data for this calculation were corrected as discussed in Results.)

VSV in presence of PPD produced 119 plaques.
VSV in absence of PPD produced 110 plaques.

Table 3

INTERFERON PRODUCTION BY RECOMBINATIONS OF SENSITIZED AND NORMAL CELLS

In Vivo Sensitizing Agent		In Vitro Challenge	Dilution of Culture Supernatant 1:5 Avg. No. of Plaques	p	1:10 Avg. No. of Plaques	p	1:20 Avg. No. of Plaques	p
Lymphs	Macros							
MT	MT	PPD	14 ± 2.9	<0.3	14 ± 1.1	<0.03	17 ± 1.7	<0.01
MT	MT	Diluent	18 ± 2.4		19 ± 1.9		28 ± 2.4	
MT	BF	PPD	10 ± 3.5	<0.001	22 ± 5.6	<0.3	12 ± 2.0	<0.01
MT	BF	Diluent	18 ± 1.3		21 ± 2.2		23 ± 1.8	
BF	MT	PPD	17 ± 1.4	<0.2	17 ± 2.4	<0.8	18 ± 2.0	<0.3
BF	MT	Diluent	13 ± 2.1		21 ± 2.7		21 ± 1.1	
BF	BF	PPD	12 ± 1.8	<0.5	12 ± 1.1	<0.2	20 ± 2.7	<0.1
BF	BF	Diluent	15 ± 2.8		16 ± 2.6		13 ± 1.7	

Abbreviations and calculations are the same as for Table 2.

VSV in the presence of PPD produced 35 plaques.

VSV in the absence of PPD produced 27 plaques.

Table 4

INTERFERON PRODUCTION RELATED TO AMOUNT OF VIRUS USED FOR INDUCTION

Cells	Concentration of Virus Added	Average No. of Plaques
CV*	10^{-1}	148
CV	10^{-2}	170
CV	10^{-3}	224
BMB**	10^{-1}	153
BMB	10^{-2}	151
BMB	10^{-3}	199

Cells = crude peritoneal cells from animals immunized with either CV or BMB.

Vesicular stomatitis virus test dose = 198 ± 30 PFU.

*Chikungunya Virus

**Baby Mouse Brain

Table 5

INTERFERON PRODUCTION BY CV-IMMUNE AND CONTROL CELLS

	Dilution of Culture Supernatants											
	1:5			1:10			1:20			1:40		
Cells*	No. of Plaques	Avg.	%	No. of Plaques	Avg.	%	No. of Plaques	Avg.	%	No. of Plaques	Avg.	%
CV	18			20			25			27		
	21			24			26			30		
	27	22	63	27	24	69	34	28	80	31	29	83
CV	11			17			18			26		
	15			22			21			31		
	15	14	40	28	22	63	29	23	66	34	30	86
CV	4			9			16			31		
	4			10			19			31		
	7	5	14	15	11	31	20	18	51	34	32	91
CV Average		14±2.6**	40		19±2.3	54		23±2.0	67		30±0.9	86
BMB	17			18			23			24		
	19			20			24			27		
	19	18	51	25	21	60	25	24	69	29	27	78
BMB	7			11			16			24		
	13			14			18			24		
	19	10	29	25	17	49	27	20	57	28	27	78
BMB Average		14±2.1	40		19±1.9	54		22±2.1	63		27±1.0	78

Footnotes for Table 5 on following page.

Footnotes for Table 5

*Crude peritoneal exudate cells from CV or BMB immunized animals incubated 24 hours with CV (10^{-1}).

Plaques counted 40 hours after L cells had been treated with supernatants and infected with VSV.

**Average plaques for combined samples of each cell type expressed ± standard error of the mean of the nine (or six) determinations at each dilution.

Percentages are based on the number of plaques formed by the test dose of VSV on untreated L cells = 35 plaques.

Table 6

INTERFERON PRODUCTION BY MACROPHAGES AND LYMPHOCYTES

In Vivo Sensitization	Cells	In Vitro Challenge	Interferon Dilution 1:7 Avg. No. of Plaques	1:14 Avg. No. of Plaques	1:28 Avg. No. of Plaques
CV	Macros	CV	6	16	36
CV	Macros	BMB	141	141	143
BMB	Macros	CV	6	17	34
CV	Lymphs	CV	72	95	109
CV	Lymphs	BMB	110	108	148*
BMB	Lymphs	CV	53	85	116

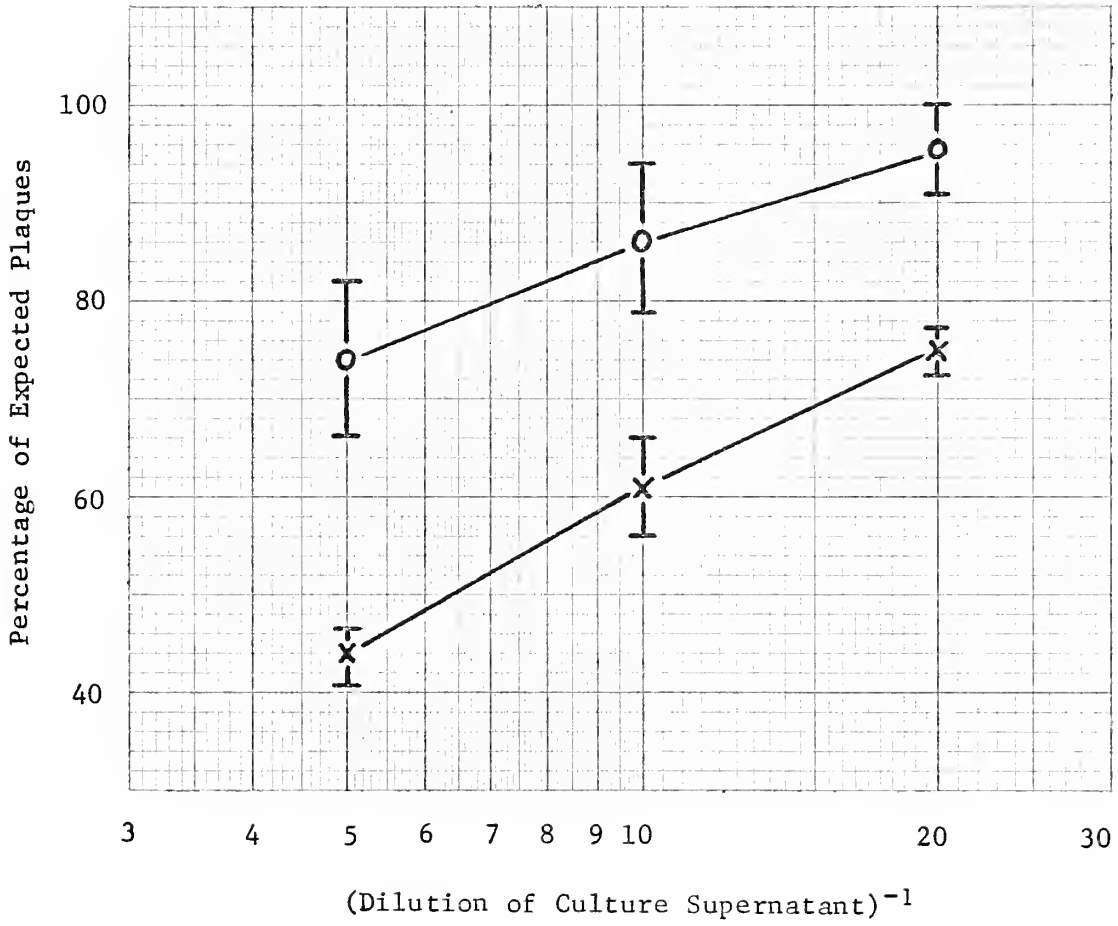
Cells from animals hypersensitized eight weeks before 24 hour in vitro challenge.
Plaques counted 40 hours after L cells had been treated with supernatants and infected with VSV.

Dose of VSV used in titration was 39 PFU.

*This average was not statistically significantly different from the averages at the other two dilutions; therefore, in calculating the percentages used in plotting the data, the average of the three dilutions was used.

Figure 1

PLAQUE INHIBITION BY MEDIA FROM THE INCUBATION OF MYCOBACTERIUM SENSITIZED CELLS WITH AND WITHOUT PPD



L cells pretreated for 24 hours with various media.

VSV plaques counted 48 hours after cells infected.

x medium from the 24 hour incubation of sensitized peritoneal cells with PPD.

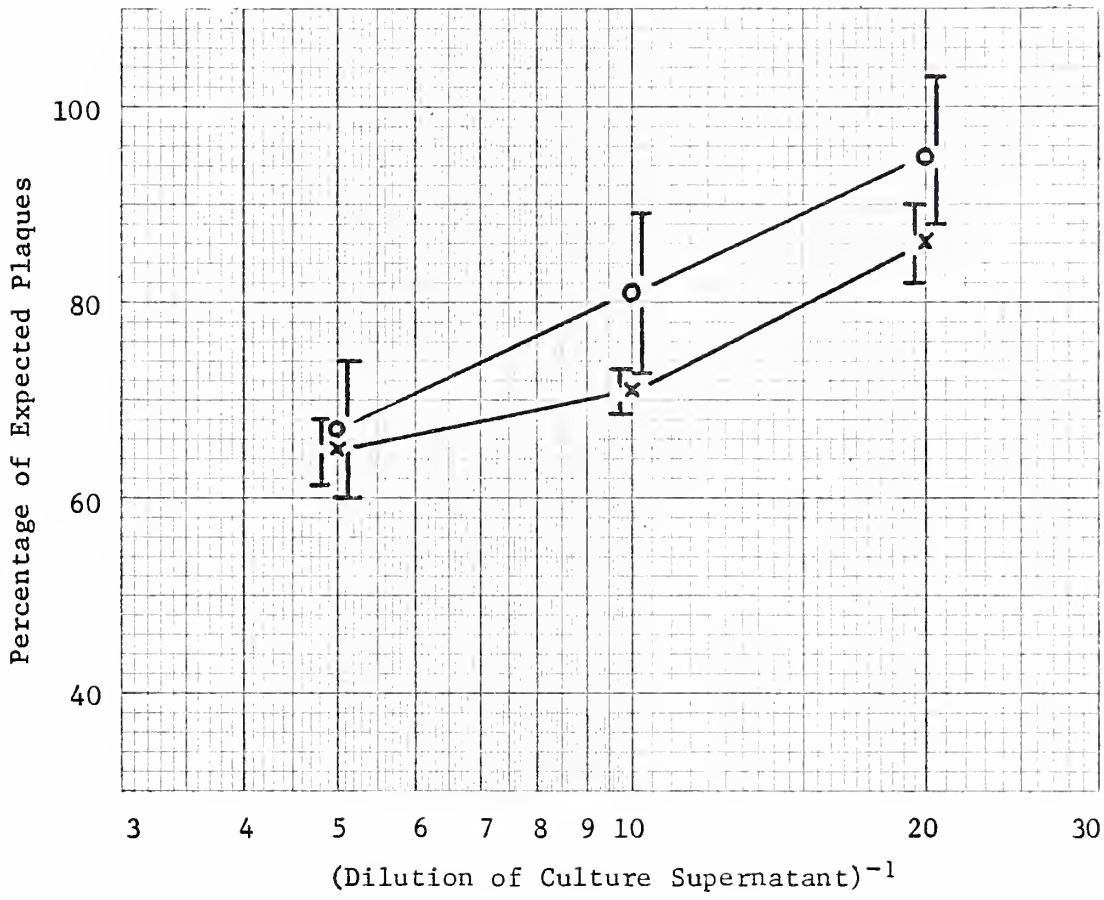
o medium from the 24 hour incubation of sensitized peritoneal cells without PPD.

Points plotted ± one standard of the mean.

Figure 2

PLAQUE INHIBITION BY MEDIA FROM THE

INCUBATION OF NORMAL CELLS WITH AND WITHOUT PPD



L cells pretreated for 24 hours with various media.

VSV plaques counted 48 hours after cells infected.

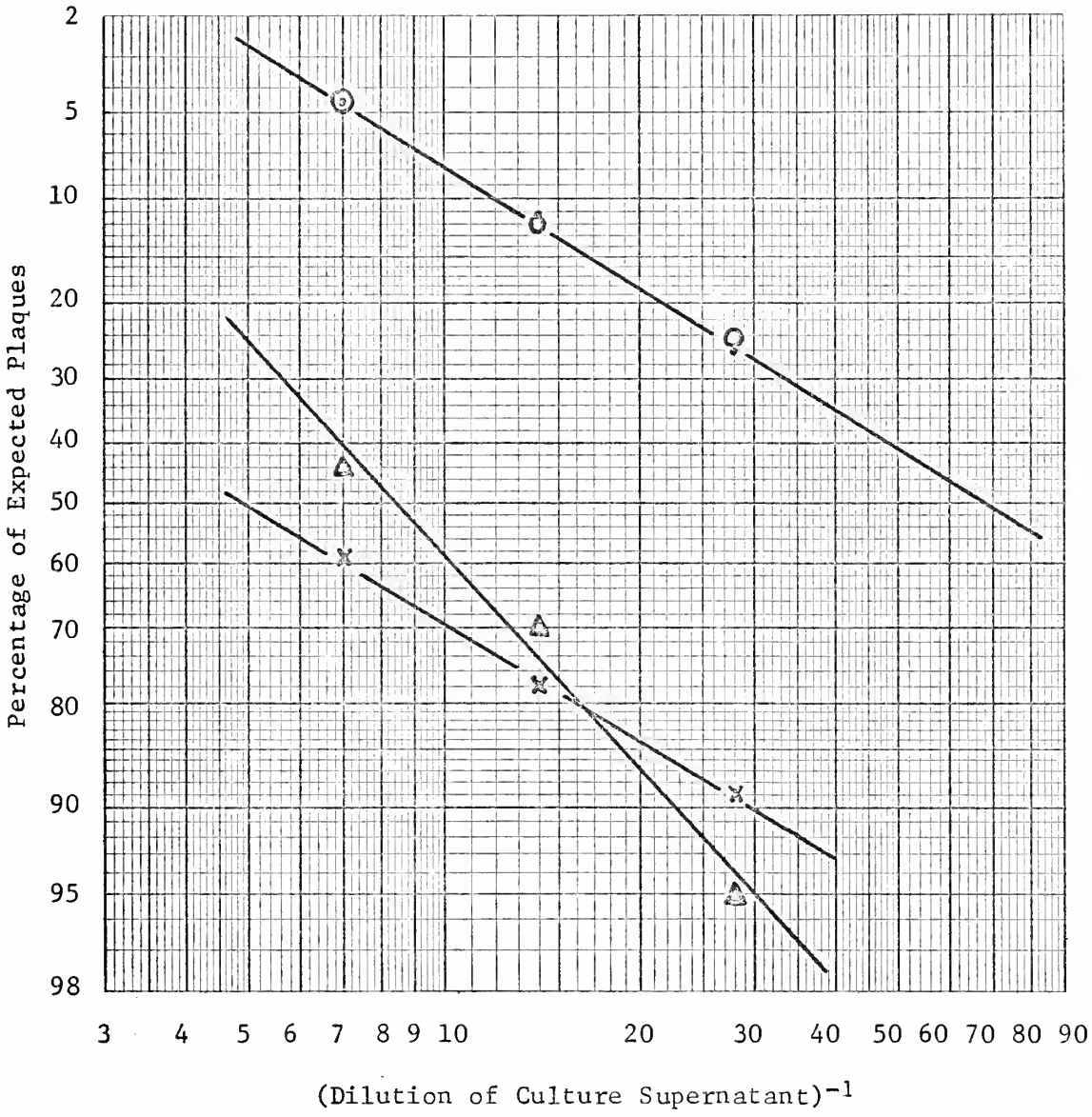
x medium from the 24 hour incubation of normal peritoneal cells with PPD.

o medium from the 24 hour incubation of normal peritoneal cells without PPD.

Points plotted ± one standard error of the mean.

Figure 3

PLAQUE INHIBITION BY MEDIA FROM THE INCUBATION OF MACROPHAGES OR LYMPHOCYTES WITH CHIKUNGUNYA VIRUS



L cells pretreated for five hours with various media.

VSV plaques counted 48 hours after cells infected.

. medium from the 24 hour incubation of CV sensitized macrophages with CV.

o medium from the 24 hour incubation of BMB sensitized macrophages with CV.

x medium from the 24 hour incubation of CV sensitized lymphocytes with CV.

Δ medium from the 24 hour incubation of BMB sensitized lymphocytes with CV.

APPENDIX

MEDIA

A. DILUENT

Medium 199 with Earles base (Grand Island Biological Co.)
2% calf serum (Flow Laboratories)
100 units/ml. potassium penicillin G (E.R. Squibb & Sons)
100 µg./ml. streptomycin sulfate (Eli Lilly & Co.)

B. CULTURE MEDIA

1. L Cell Growth Medium

Minimum Essential Medium (Eagle's) with Earles base (Grand Island Biological Co.)
10% calf serum
100 units/ml. potassium penicillin G
100 µg./ml. streptomycin sulfate

2. Peritoneal Exudate Harvesting Medium

Medium 199 with Earles base or Roswell Park Memorial Institute Medium 1640 (Microbiological Associates, Inc.)
20% fetal calf serum heat inactivated for 30 min. at 56°C. (Grand Island Biological Co.)
10 units/ml. sodium heparin (Organon, Inc.)
100 units/ml. potassium penicillin G
100 µg./ml. streptomycin sulfate

3. Peritoneal Exudate Maintenance Medium

Roswell Park Memorial Institute Medium 1640
20% fetal calf serum heat inactivated for 30 min. at 56°C.
100 units/ml. potassium penicillin G
100 µg./ml. streptomycin sulfate

4. First Agar Overlay

(a) Medium 199 with Earles base -2X
4% calf serum
200 units/ml. potassium penicillin G
200 µg./ml. streptomycin sulfate

(b) 2 gm. Ionagar No. 2 (Oxoid)
100 ml. distilled water

Warm solution (a) to 37°C. in water bath. Cool freshly autoclaved solution (b) to 56°C. in water bath. Mix equal volumes of solutions (a) and (b) and use within 20 minutes.

5. Second Agar Overlay

Prepare as first agar overlay except that solution (a) also contains 67 µg./ml. neutral red (National Aniline Division, Allied Chemical)

Note: Minimum Essential Medium with Earles base, Medium 199 with Earles base, and Medium 199 with Earles base -2X were prepared from pre-mixed powders (Grand Island Biological Co.)

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